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14. ABSTRACT
Spinal cord injury (SCI) exacts an enormous social and financial burden on society. As such, there has been considerable attention directed at finding treatment strategies, including development of tissue and cell transplant techniques. However, the current approaches do not adequately address the complexity of the injury site, such as lesion length and an environment that is usually non-permissive for axon regeneration. We have developed tissue engineered nerve grafts (TENGs) consisting of living dorsal root ganglia (DRG) and axons that can be stretch-grown to a length necessary to bridge extensive lesions. In current studies, we have optimized in vivo laminoplasty procedures, transplanted 5 mm–long stretch-grown constructs into a 5 mm-long lesion in the rat thoracic spinal cord, and have demonstrated survival of DRGs up to 6 weeks post-transplant. If successful, this approach will provide an alternative or additional means to repair large spinal lesions.

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INTRODUCTION

Spinal cord injuries (SCIs) have become a common consequence of motor vehicle crashes, falls, and acts of violence (most typically gunshot wounds). Following SCI, the central nervous system (CNS) is not able to promote enough axonal regeneration across the injury site to fully restore functional deficits. After the primary mechanical insult that causes the SCI, a complex process of secondary damage occurs within the cord due to inflammation, ischemia, and apoptosis, among other processes with distinct pathophysiology including the formation of cysts, cavitation, and a highly reactive glial scar. Many severed axons initially sprout (1), however they retract as they have a poor intrinsic capacity for growth and encounter a series of inhibitory factors that are nonpermissive for growth, including proteoglycans such as chondroitin sulfate proteoglycans (CSPGs), which contribute to the glial scar.

Several models have been used in order to best mimic the primary and secondary events occurring in human spinal cord injury. These include a contusion or compression to the cord, a transection through the cord, and a transection and evacuation of the cord. Complete transection models are considered the "gold standard" to test regeneration—providing the potential to definitively show regeneration of transected axons through a lesion, rather than sparing or sprouting of intact axons (2). In complete transection models, a gap results from spinal cord retraction (in transection-only models) or retraction and tissue removal (in transection and evacuation models). In rat models, the largest complete transections and evacuations are generally 4-5 mm (3-12), but models with up to 10 mm long complete evacuations have been used (12-16).

In order to bridge these gaps and promote nerve repair, a bridge must be implanted in the gap. A method must be used to provide an 'on-ramp,' attracting axons into the bridge; provide surfaces on which the axons can grow across the bridge, with sufficient physical adhesion to permit that growth, yet not too much adhesion, which would prevent growth. Once the axons have grown across the lesion, there must be a means of an 'off-ramp,' that coaxes axons to cross the distal glial scar along the distal margin of the lesion to enter viable tissue and form connections (17).

Despite widespread findings that the CNS environment is inhospitable to axon regeneration, Silver and colleagues showed that transplanted dorsal root ganglion cells (DRGs) can grow robustly in the adult CNS (18). He used a unique microinjection technique in order to minimize scarring and injected dissociated adult DRGs rostral to a dorsal column transection of the spinal cord. From the sensory neurons, robust regeneration occurred in both directions at a rate of 1 mm per day, even when delivered chronically, and even through regions of reactive astrocytes and degenerating myelin. However, growth cones abruptly aborted at high concentrations of CSPGs at the lesion epicenter. These findings suggest that, if DRG transplants can surmount local scarring in a spinal cord lesion, they may have the potential to extend processes that help promote and facilitate the extension of host axons along their own axonal tracts.

In the current effort we used tissue engineered nerve grafts (TENGs) to bridge spinal cord lesions (Figure 1). TENGs are the first ever living engineered nervous tissue based on the process of stretch growth, a naturally occurring process through development that we are able to recapitulate *in vitro* using custom built mechanobioreators. In short we isolate two populations of dorsal root ganglia sensory explants (DRGs) and plate them along an interface. One population is on a stationary substrate while the second is on a movable substrate. Using a stepper motor system, we can slide back the moving substrate with the population of DRGs in micron-size increments thus stretching or elongating the axonal tracts spanning the two populations of neurons. This mechanical process induces extreme stretch growth of the center portion of the axon that can reach at least 1 cm of growth per day and extent at least 10 cm in length.

We propose that TENGs may create new intraspinal circuits across SCI lesions. When used as a delayed treatment for a 10 mm long modified lateral hemisection (Th9-Th11), we found TENG survival at 4 weeks using cell tracking methods and immunohistochemistry (19). Delaying transplantation until after the event of acute trauma is more clinically relevant. In addition, cells and tissue have been shown to survive better in transection injuries when transplanted at a sub-acute time point rather than acutely (20,

21). In a study of fetal spinal cord transplants with neurotrophin administration, supraspinal axons grew through the transplant and into the caudal host spinal cord in 89% of animals when transplanted 2 weeks after thoracic transection but in only 55% of animals when transplanted acutely (21).

In this study, we evaluated TENGs ability to promote spinal cord regeneration in a 5 mm complete transection model in order to more definitively test whether axon regeneration was occurring through our grafts. Through H&E staining and immunohistochemistry, we found that TENGs survive out to 6 weeks post-implantation, with their axons extending into and through glial scar early on. H&E staining shows that animals treated with TENGs had an injury site with more dense tissue infiltration and less compression. Additionally, early evidence demonstrates TENG axons ability to grow and extend out caudally into host tissue. In this study we also looked at the effect the TENGs had on glial scar formation, cyst formation, and immune response. If able to address these universal challenges associated with SCI, TENGs may offer an alternative treatment option to the conventional methods of stem cell delivery and three-dimensional scaffolds.

BODY

Specific Aim of the Work: Histological and behavioral evaluation of effects of transplanted nervous tissue constructs in a 5 mm model of complete spinal cord segment excision (T9-T11) at 10 days, four weeks, and six weeks post-repair. We now transplant tissue engineered nerve grafts (TENGs) consisting of long axonal tracts (5 mm long) and two populations of dorsal root ganglia (DRG) to bridge an excised segment (also 5 mm in length) of the rat thoracic T10-T11 spinal cord (Figure 2). A primary injury is performed to remove the cord and fill the cavity with a collagen only hydrogel. The TENG is then transplanted during a secondary surgery, 10 days after the initial injury. During this surgery, the initial hydrogel is carefully removed and the TENG is transplanted within a new collagen hydrogel. All TENGs are virally transduced to express green fluorescent protein (GFP) with an AAV viral vector (AAV2/1.hSynapsin.EGFP.WPRE.bGH, UPenn Vector Core) to permit *in vivo* identification. Experimental groups include animals that receive the collagen only hydrogel during the secondary injury or animals that receive a TENG embedded in the collagen hydrogel. Furthermore, groups are divided into animals that do or do not receive passive rehabilitation. The passive rehabilitation is an exercise regimen using motorized bikes that is performed three times per week for 60 minute sessions (with a 10 minute break). We also perform weekly functional outcome assessments using the Basso, Beattie and Bresnahan (BBB) locomotor scale to evaluate potential recovery of motor function.

We have performed extensive histological examinations on constructs at 10 days, 4 weeks, and 6 weeks post-transplant. Upon harvest of the spinal cord tissue, columns were dissected and post-fixed overnight in 4% paraformaldehyde, and then spinal cords were dissected from spinal columns and cryoprotected in 30% sucrose. A 3-4 cm long segment was cut surrounding the lesion, embedded in O.C.T. medium, and flash frozen in isopentane. Parasagittal longitudinal sections (20 μm thick) were cut on a cryostat (Leica CM1850) and mounted on Superfrost™ Plus slides. Representative sections were stained with hematoxylin and eosin (H&E) using standard procedures.

Fluorescent immunohistochemistry was performed using the following primary antibodies: mouse anti-SMI31 (1:1000, Covance), mouse anti-SMI32 (1:1000, Covance), chicken anti-Glial Fibrillary Acidic Protein (GFAP; 1:250, Millipore), rabbit anti-Calcitonin Gene Related Peptide (CGRP; 1:1000; Sigma), and rabbit anti-Iba-1 (1:1000, Wako). Slides were rinsed in PBS, blocked in 2% normal horse serum with 0.3% Triton X-100, and incubated in blocking buffer with primary antibodies overnight at 4°C. Slides were rinsed in PBS with 0.05% Tween and then incubated with the appropriate Alexa Fluor®-conjugated secondary antibodies (diluted 1:500 in PBS with Tween and 5% normal horse serum) for one hour at room temperature. Slides were rinsed and then coverslipped with Dako fluorescent mounting medium.

Fluorescent microscopy was conducted using a Nikon Eclipse microscope. Spinal cord lesion thickness and cyst area were measured in H&E-stained longitudinal sections using Aperio Webscope Spectrum. Lesion thickness was measured at the rostrocaudal midpoint of the injury site in two midline sections per animal. In the same sections, cyst area was measured by encircling cystic regions devoid of tissue rostral and caudal to the injury site.

Following spinal cord transection, motor function was assessed weekly using the Basso, Bettie, and Bresnahan (BBB) scale (22). Briefly, each animal was allowed to move around freely in an open field (75 cm x 95 cm) for four minutes while two trained scorers jointly assessed the movement of each hindlimb on a scale from 0 (no movement) to 21 (normal). All animals were also tested prior to injury.

Results: Our 5 mm long complete evacuation model (Figure 2) has been successful in minimizing connective tissue infiltration as well as preventing compression of the spinal cord and transplant. TENGs are consistently robust and brightly fluorescent at the time of transplantation (Figure 1D). Additionally, TENGs remain viable even though animals do not receive any immunosupressants. At 10 days following transplant, 100% of TENG transplants survived (*n=4*) (Figure 3). In 2 out of 4 cases, TENG survival was robust, with TENG axons extending throughout the lesion cavity and some TENG axons penetrating host tissue both rostrally and caudally. This high TENG viability is observed at 6 weeks

(Figure 4) post-transplant also with GFP+ TENG bodies present throughout the full rostrocaudal length of the lesion and GFP+ axons extending longitudinally and laterally to completely fill the lesion cavity. Furthermore, our findings provide evidence of host axon integration with and along TENG axons (Figure 5), a phenomenon our group has previously observed in peripheral nerve injuries treated with TENGs. In several cases we observed TENG axon growth into stumps and through GFAP+ areas, with one animal demonstrating growth up to 4.8 mm into a dorsal rootlet and several showing deep growth into host spinal cord (maximum case was observed to be 1.7 mm).

Histological findings from this study demonstrated several challenges that exist with this full transection model including a dense, GFAP+ glia scar, cysts, compression of the cord at the site of injury, and cyst formation over time (Figure 6). In the case of compression, there was a trend towards collagen controls exhibiting thinner cord thickness than TENGs. There were large cysts present in the rostral and caudal host stumps in many cases, generally more frequent and larger in size at later timepoints. No difference was observed in cyst area between groups. We also observed immune response in each case via Iba-1 staining. Iba-1+ cells were found to have highly activated, ameboid morphologies throughout the lesion area and in both rostral and caudal host stumps. This was the case in both TENG transplants and collagen controls at 10 days, 4 weeks, and 6 weeks. In all cases examined, morphologically less activated Iba-1+ cells could be found only distant to the lesion area, well away from any cystic regions.

Functional recovery was recorded via BBB testing. All animals scored 21 on the BBB scale prior to injury, indicating normal hindlimb function. After transplant, animals scored between 0.5 and 7 for the duration of the study, indicating a range of hindlimb joint movement but no plantar placement or weight support. As expected by the relatively early post-injury time points tested, no significant differences in BBB scores were found between TENG transplants and collagen controls.

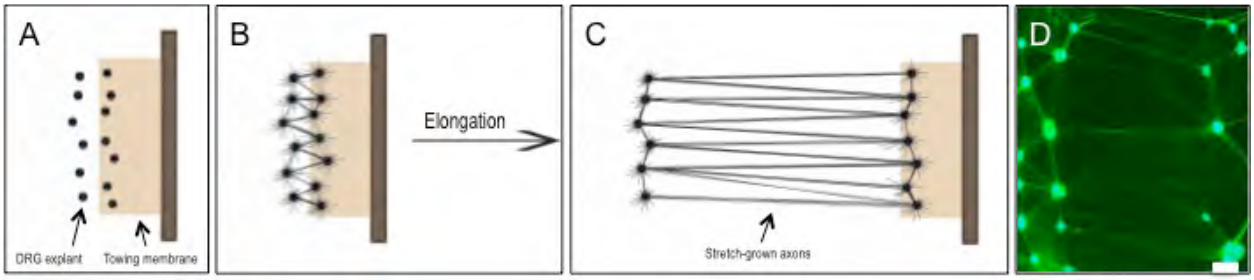

Figure 1: Procedure for TENG generation. (A) Placement of DRG explants along the plastic interface in the bioreactor after isolation. Each black dot represents one DRG explant. One population is plated on the base (left - stationary side) and one on the towing membrane (right - movable substrate). **(B)** Axonal networks form between two DRG populations after five days in culture. Mechanical tension, "elongation", is applied to the one population plated on the towing membrane. **(C)** Stretch-grown axonal tracts form between DRG populations under continuous mechanical tension. **(D)** Example of a stretch-grown TENG transduced to express green fluorescent protein (GFP). Scale bar = 1mm.

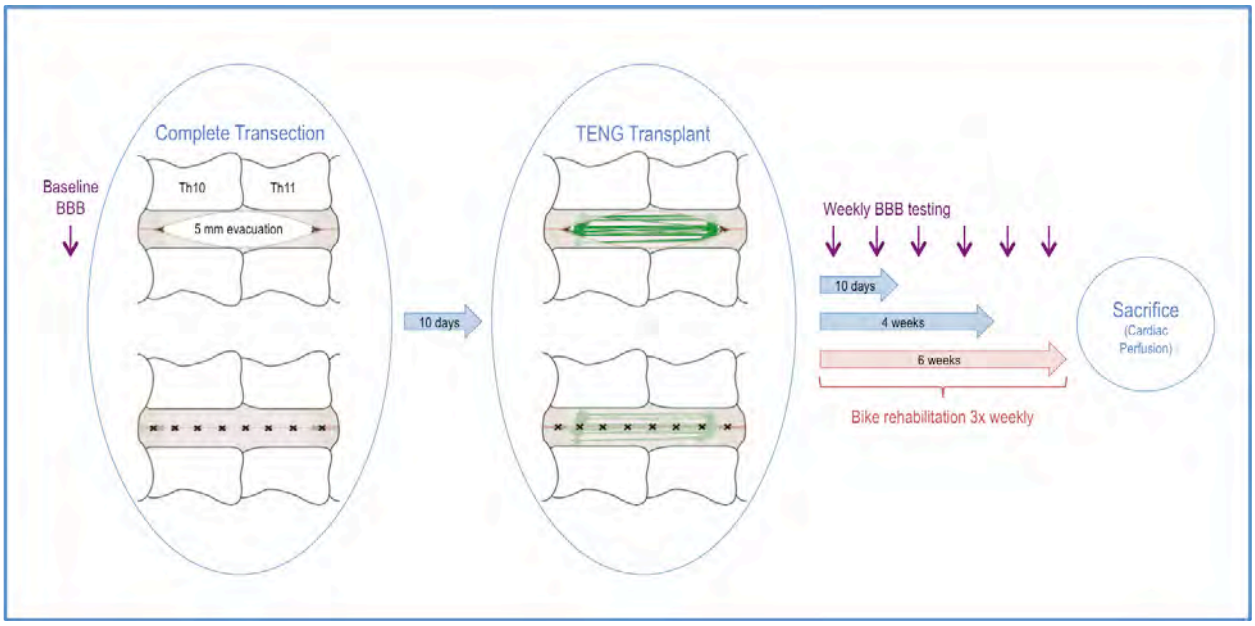

Figure 2: Rodent Spinal Cord Injury Model and Experimental Design. (Left to right) The initial spinal cord injury consists of a 5.0 mm transection and evacuation of the cord at the levels of T10-T11. Collagen hydrogel is inserted into the cavity and a baseline BBB score is recorded. The injury site is sutured close. At ten days post-initial injury, the cavity is reopened, the hydrogel is removed, and a 5.0 mm gap exists. GFP+ TENGs (5.0 mm in length) stretch grown in culture are encapsulated in a collagen type I hydrogel and rolled into the cavity site, bridging the spinal cord injury. Post injury and replacement with either a TENG or a collagen hydrogel control, the dura is sutured together for protection and prevention of invading tissue. Post-repair a weekly BBB score is recorded. Bike rehabilitation is performed three times weekly through the final time points of ten days, four weeks, and six weeks at which point a sacrifice is performed via cardiac perfusion.

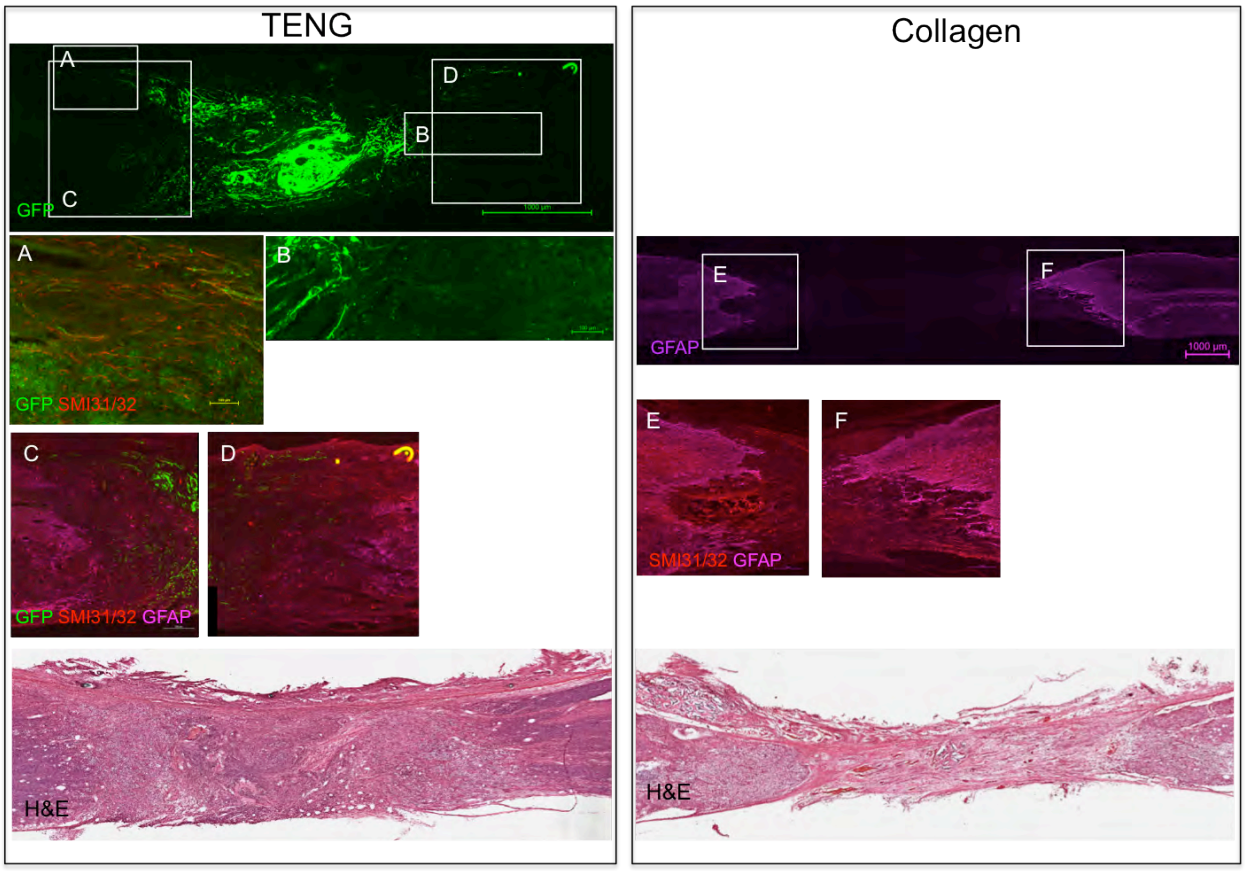

Figure 3: Histological Examination at Ten-Days Post-Repair: Survival of GFP-positive TENG vs. Treatment with Collagen Only. (A-D) Longitudinal frozen sections of rat TENGs (green) surviving (absent immunosuppression) and maintaining their morphology both in neuronal bodies and axonal architecture at 10 days post transplant. SMI31/32 (red) antibody immunostains all axons, both thick and thin. GFAP (glial fibrillary acidic protein) antibody identifies astrocyte cells. H&E image demonstrates thickness of injury site area in animals treated with TENGs vs animals treated with collagen only. **(A)** Magnified rostral region demonstrating TENG GFP+ axons entering host, SMI31/32+ tissue. **(B)** Magnified caudal region demonstrating outgrowth of TENG GFP+ axons penetrating into distal stump at this early timepoint. **(C & D)** Magnified rostral and caudal stumps respectively showing GFAP+ glial scar, GFP+ TENG axons entering through glial scar, and SMI31/32+ host axons interacting with TENG axons. **(E-F)** Longitudinal frozen sections of animals treated with collagen only. Thick glial scars are indicated by a high degree of GFAP staining on the proximal and distal ends of the injury site. H&E image demonstrates relatively thinner injury site area in animals treated with collagen only. **(E&F)** Magnified rostral and caudal stumps respectively showing GFAP+ glial scar and SMI31/32+ host axons.

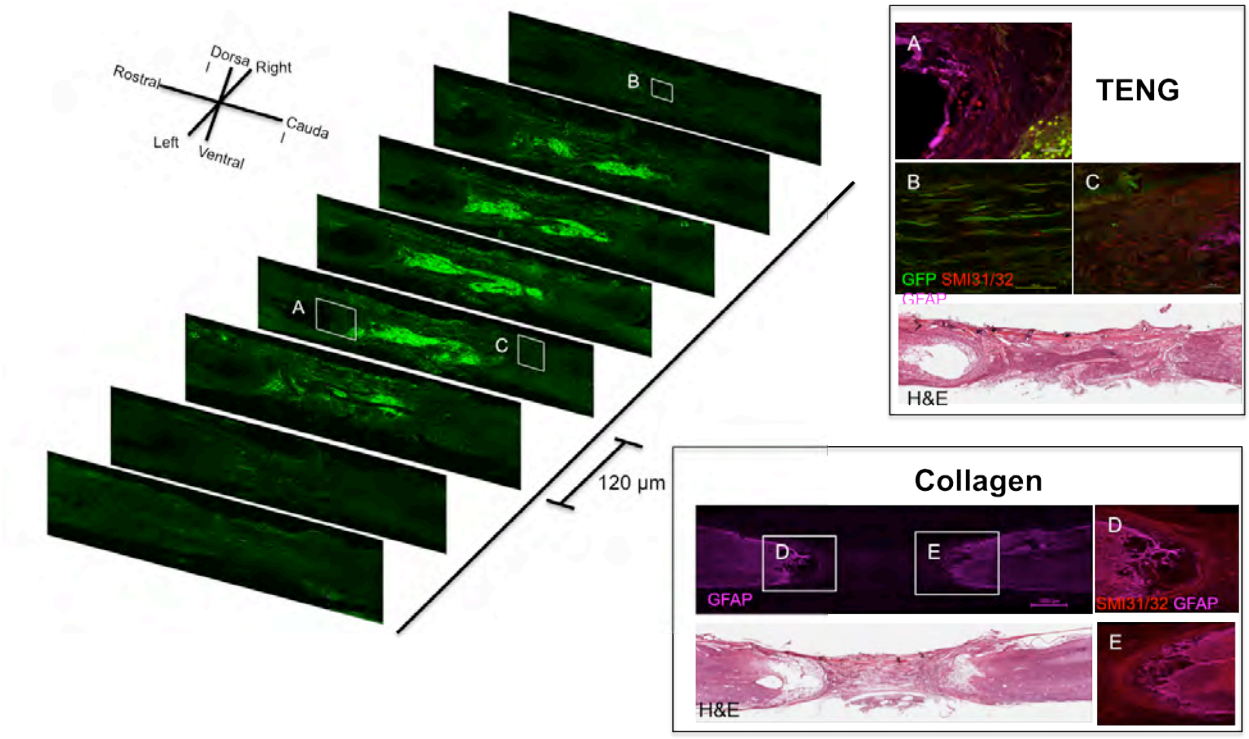

Figure 4: Six-week Survival of GFP-positive TENG or Collagen Only. (Left) Three-dimensional reconstruction of a GFP +TENG (green) at six weeks post-transplantation. **(Right)** Longitudinal frozen sections and H&E stained sections of rat TENGs (green) (**A-C)** or collagen only (**D-E)** transplants. SMI 31/32 (red), GFAP (purple). **(A)** Magnified rostral region demonstrating TENG GFP+ bodies, SMI31/32+ tissue at the GFAP+ glial scar. **(B)** Magnified middle region demonstrating interactions between TENG GFP+ axons and host SMI31/32+ axons. Preliminary evidence of axon induced axonal growth. **(C)** Magnified caudal stump showing GFAP+ glial scar, GFP+ TENG axons entering through glial scar, and SMI31/32+ host axons interacting with TENG axons. **(D&E)** Magnified rostral and caudal stumps respectively showing GFAP+ glial scar and SMI31/32+ host axons.

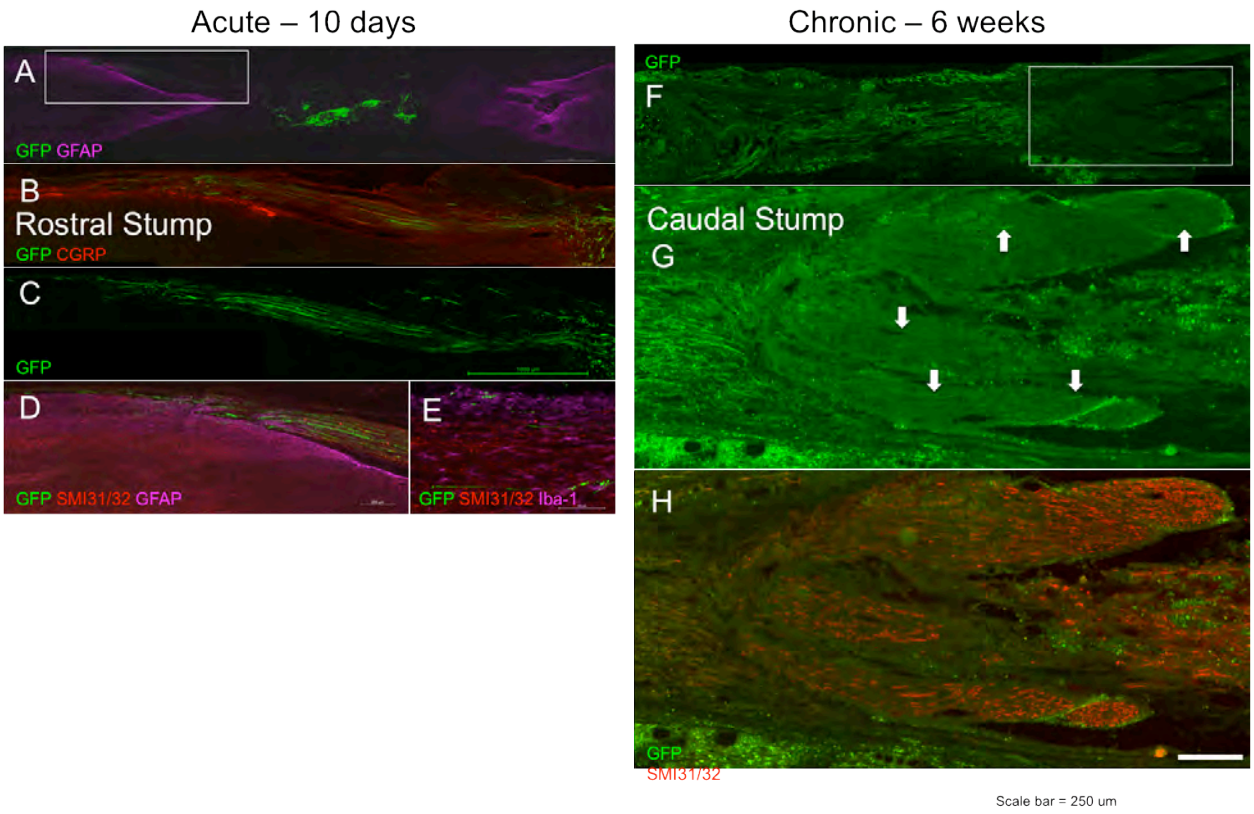

Figure 5. TENG Outgrowth Indicating Possible Host Axon Growth Along TENG Axons at 10 days and 6 weeks Post-Repair. (A) Longitudinal section of GFP+ TENG (green) at 10 days post-transplantation. Immunohistochemistry shows GFP+ TENG (green) within cavity and GFAP+ proximal and distal stumps. **(B)** Magnified region outlined in (A) showing rostral stump. GFP+ TENG (green) axons penetrate deeply into CGRP+ (red) dorsal rootlet. (**C)** GFP+ TENG (green) axons penetrating into dorsal rootlet as in image (B). **(D)** Dorsal rootlet in (B) re-entering spinal cord. **(E)** Magnified region in the middle of the construct showing Iba-1 immune reactivity (purple). **(F)** Longitudinal section of GFP+ TENG (green) at 6 weeks post-transplantation. Immunohistochemistry shows GFP+ TENG (green) within cavity. (**G)** GFP+ TENG (green) axons penetrating into caudal stump as outlined in image (F). White arrows point to TENG axon extensions. **(H)** GFP+ TENG (green) axons penetrating into caudal stump as outlined in image (F) interacting with host SMI31/32+ (red) axons.

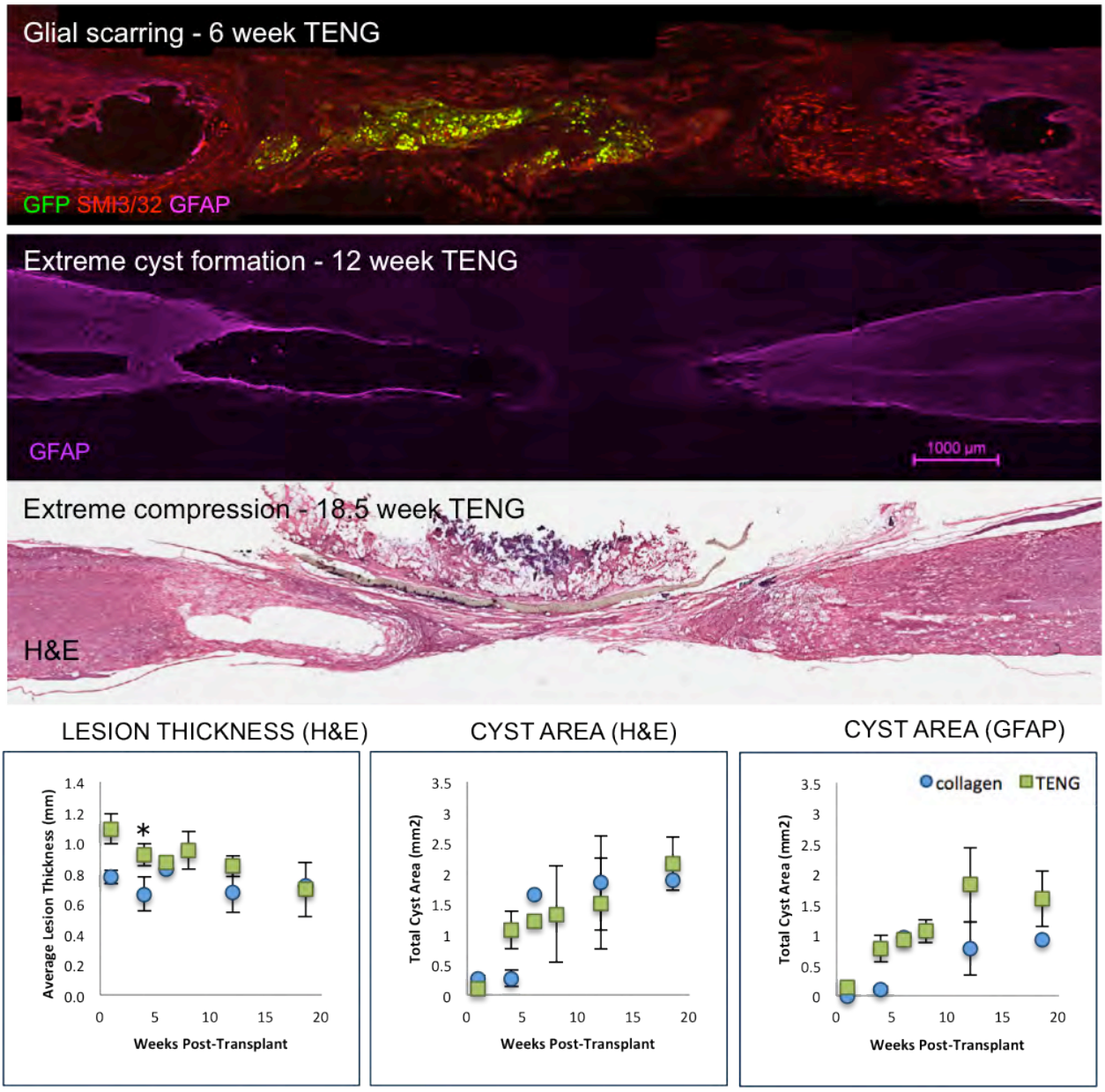

Figure 6. Challenges: scarring, cysts, & compression (Top, Middle, Bottom) Longitudinal frozen and H&E stained sections demonstrating some of the challenges that exist with this model. Glial scarring (top image) as demonstrated by high degree of GFAP staining on both the proximal and distal ends of the injury site. Extreme cyst formation (middle image) occurred in several animals. H&E image (lower image) at 18.5 weeks post-repair demonstrates extreme compression of injury site in several animals. Quantification of lesion thickness as recorded from H&E stained images, cyst area as recorded from H&E stained images, and cyst area as recorded from GFAP stained images.

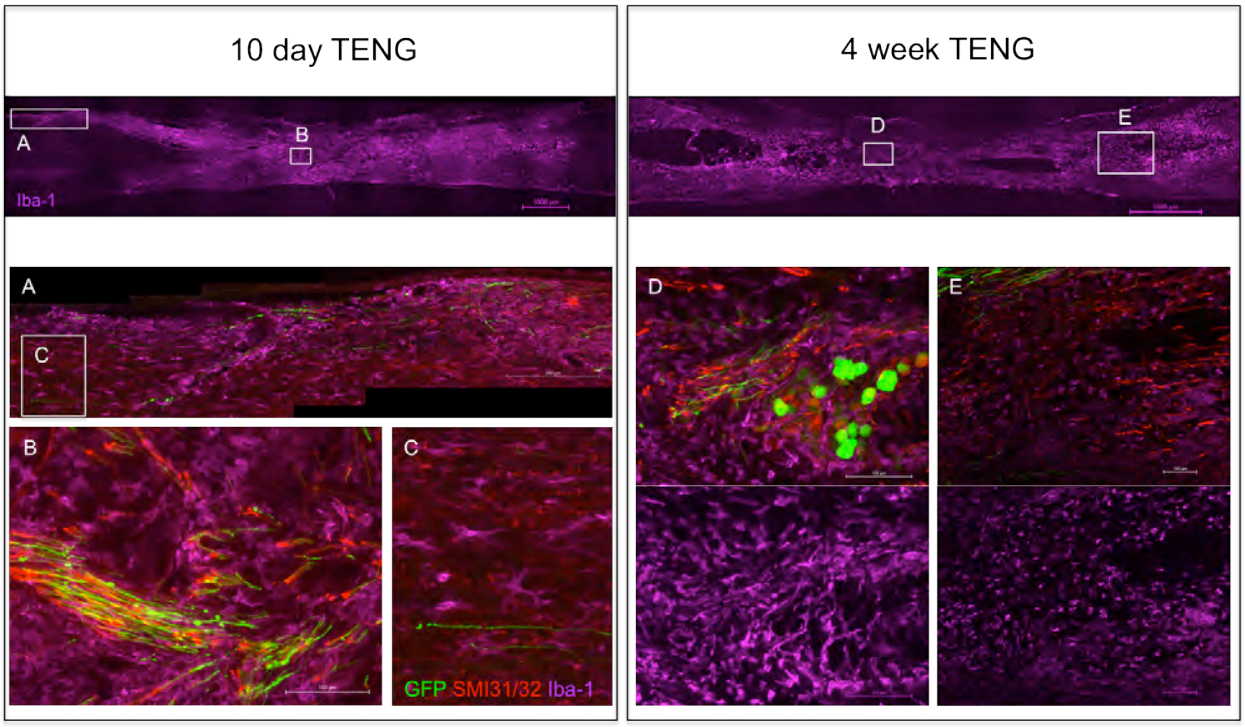

Figure 7. Immune Response as Indicated by Iba-1 Reactivity at 10 days and 4 weeks Post-repair with TENG. (A-C) Longitudinal sections at 10 days post-repair with GFP+ TENG. **(D-E)** Longitudinal sections at 4 weeks post-repair with GFP+ TENG.

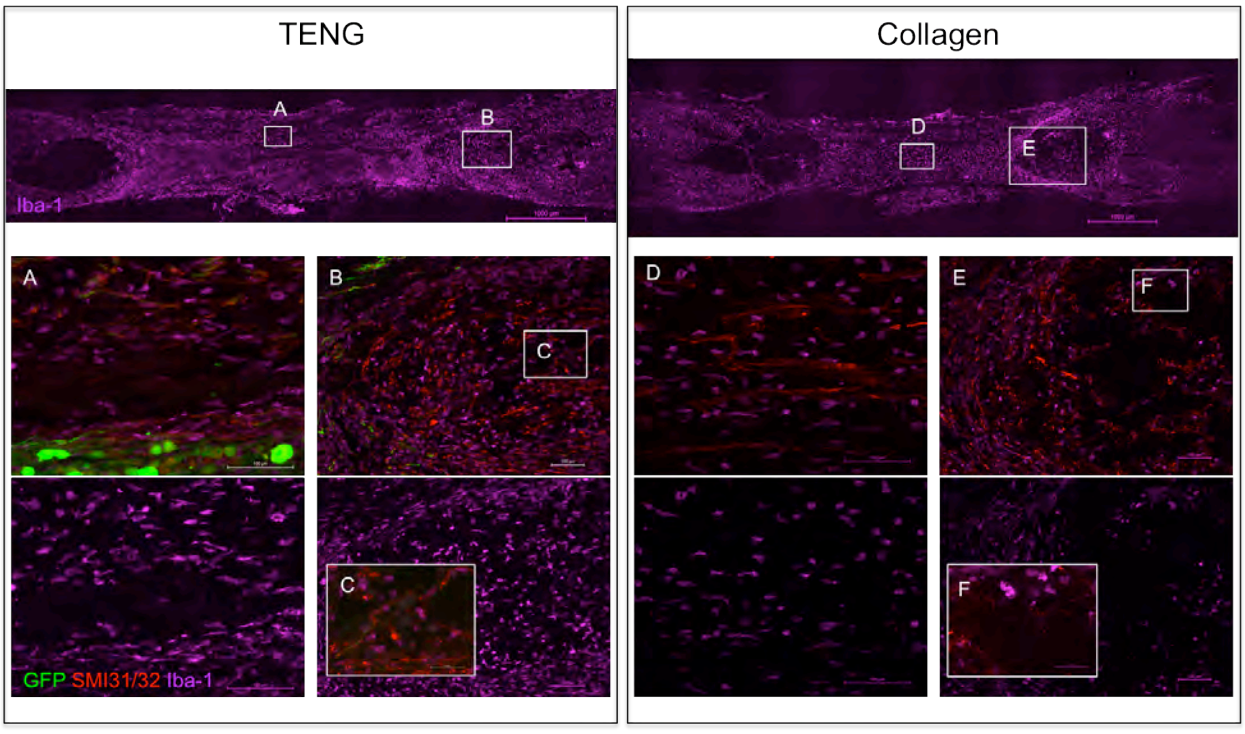

Figure 8. Immune Response as Indicated by Iba-1 Reactivity at 6 weeks Post-repair with TENG. (A-C) Longitudinal sections post-repair with GFP+ TENG. **(D-E)** Longitudinal sections post-repair with collagen only.

Challenges and Future Directions:

Although a complete transection is considered the "gold standard" for demonstrating regeneration, it is widely acknowledged to be a challenging model (2). Others have faced challenges in transitioning from a partial lesion to a full transection model, including problems with transplant survival (23). Therefore, future studies will investigate methods in order to mitigate challenges faced with the current full transection model specifically to address problems associated with glial scarring, cord compression, and cyst formation. Increasingly, the consensus in the field of spinal cord injury repair is towards the need to use combinatorial treatment strategies (24-26). With that said, in the future we will look into using immunosuppression in order to increase TENG viability at longer time point (> 6 weeks) (27, 28), chondroitinase ABC to break down the glial scar (4, 5, 24, 29-31), using the TENG as an external bridge (30), conditioning lesion/scar removal for delayed transplant (21, 32-34), as well as anti-inflammatory treatments (35).

KEY RESEARCH ACCOMPLISHMENTS

- Developed a method to evaluate the efficacy of stretch grown axons to enhance recovery posttraumatic spinal cord injury.
- Demonstrated survival and maintenance of axonal architecture of TENGs at 10 days and up to 6 weeks post-transplant, without immunosuppression.
- Observed evidence of TENG axons extending and penetrating deep into host tissue but rostral and caudal to the site of injury. Sometimes, the TENG axons were able to penetrate through GFAP+ glial scarring.
- H&E staining revealed differences between cord thicknesses amongst different treatment conditions. Animals treated with TENGs trended towards thicker spinal cords as compared to animals treated with collagen only.
- Observed preliminary evidence of axon-induced axon regeneration across TENGs in as little as 10 days and out to 6 weeks post-transplant. If proven true, this finding would confirm findings our group has found with TENGs in peripheral nerve models of repair.
- Identified areas to improve in the full transection model in order to enhance the effects of TENGs at bridging severe spinal cord injury.

REPORTABLE OUTCOMES

In this study, we evaluated the ability of TENGs to promote spinal cord regeneration in a 5 mm complete transection model in order to more definitively see if axon induced axon regeneration was occurring through our grafts. Through H&E staining and immunohistochemistry, we found that TENGs survive out to 6 weeks post-implantation, extending into and through glial scar early on. H&E staining shows that animals treated with TENGs had an injury site with more dense tissue infiltration and less compression. Additionally, early evidence demonstrates TENG axons ability to grow and extend out caudally into host tissue. We also observed the effect the TENGs had on glial scar formation, cyst formation, and immune response. If able to address these universal challenges associated with SCI, TENGs may offer an alternative treatment option to the conventional methods of stem cell delivery and three-dimensional scaffolds.

CONCLUSION

Spinal cord injury (SCI) is a major source of morbidity and mortality in the United States. As such, there is a need to identify methods of treatment which will improve recovery in patients suffering from SCI and reduce the severity of long-term sequalae. The goals of this experiment were to evaluate the effects of transplantation of neural cells, TENGs, on functional recovery after rats are subjected to spinal cord injury. We propose that by using TENGs we may be able to create new intraspinal circuits across SCI lesions. In the current work we have evaluated our TENGs in a 5.0 mm full transection SCI model and have found robust survival and evidence of nerve regeneration across our TENGs out to 6 weeks post-transplantation. TENGs in this study also demonstrated preliminary results indicating axon-induced axon regeneration, a phenomenon we believe is not possible with a collagen hydrogel treatment only. TENG axons penetrate deeply into the rostral and caudal host stump even in the presence of glial scarring. Using this extreme model of 5 mm long lesion of complete spinal cord tissue removal, we faced several anticipated and unanticipated challenges. As expected, substantial glial scarring occurred along the margins of the SCI lesion and due to the substantial length of the lesion, substantial spinal cord compression was observed. However, with this first 6 week examination of such a large tissue evacuation, we found cyst formation beyond the original margins of the lesion, both rostrally and caudally. This unforeseen cyst formation appeared to be the biggest obstacle for the continuation of the host regeneration across the lesion seen at earlier time points. Considering that the results collected thus far support the hypothesis that TENGs enable regeneration across nerve lesions following SCI, a refined approach is warranted. Accordingly, we have developed strategies to mitigate the lesion expansion for future studies.

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